A Simultaneous Assessment of CYP3A4 Metabolism and Induction in the DPX-2 Cell Line

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ABSTRACT

The DPX-2 cell line, a derivative of HepG2 cells, harbors human PXR and a luciferase-linked CYP3A4 promoter. These cells were used in a panel of cell-based assays for a parallel assessment of CYP3A4 induction, metabolism, and inhibition at the cellular level. CYP3A4 induction in the DPX-2 cell line by various agents was monitored in 96-well plates by a luciferase-based transcriptional activation assay. Of the prototypical CYP3A4 inducers examined, all exhibited elevated luciferase activity in DPX-2 cells. CYP3A4 enzyme activity in noninduced and rifampicin-induced DPX-2 cells was also assessed using Vivid fluorogenic substrates. Significantly elevated CYP3A4 activity levels (2.8-fold ± 0.2-fold above DMSO-treated cells) were found in DPX-2 cells after 48 hours of exposure to rifampicin, but were undetectable in parental HepG2 cells. Rifampicin-induced activity levels were found to be suitable for assessing the inhibitory potential of new chemical entities in downstream CYP3A4 inhibition assays. The elevated CYP3A4 activity was inhibited 85% by 10 μM ketoconazole. In addition, a cytotoxicity assay to correct for possible toxic effects of compounds at the cellular level was applied. The comparative data obtained with a combination of the above assays suggests that the application of several independent in vitro technologies used in DPX-2 cells is the best possible strategy for the assessment of the complex phenomena of CYP3A4 induction and inhibition.

KEYWORDS: CYP3A4, induction, inhibition, DPX-2 cell line, drug-drug interactions

INTRODUCTION

CYP3A4 is a critical member of the CYP3A subfamily of cytochrome P450 enzymes and is involved in the metabolism of more than half of all currently used drugs. Furthermore, this P450 is implicated in several well-documented cases exhibiting clinically important drug-drug interactions and toxicities related to CYP3A4 inhibition.[1] For this reason, an ability to quickly obtain and accumulate reliable data to evaluate the ability of compounds to interfere with CYP3A4 metabolism is considered to be critical for the modern drug discovery process. Currently, several in vitro assays to screen for CYP3A4 inhibition both in high-throughput and low-throughput formats have been developed and commonly used at different stages of drug development.[2-4] Coincidently, toxicological and pharmacological implications of CYP3A4 induction remain much less investigated. Recent reports indicate that altered expression of this enzyme may be of clinical significance and could result in increased toxicity due to accumulation of toxic metabolites, drug side effects, or by altering the therapeutic efficacy of a coadministered drug.[5,6] Therefore, identifying compounds involved in CYP3A4 induction, in addition to CYP3A4 inhibitors, would allow broadening the overall prediction of the potential for CYP3A4-related toxicities and drug-drug interactions.

CYP3A4 is a highly inducible enzyme with modulators that belong to a chemically diverse group of compounds consisting of drugs, steroids, and various nutraceuticals including herbal preparations.[7,8] CYP3A4 induction is a time-dependent and concentration-dependent phenomenon resulting in increased levels of enzyme. For the most part, induction of CYP3A4 involves pregnane X receptor (PXR), a transcription activation factor present in the cytosolic fraction of cells and identified as one of the key upstream modulators of CYP3A4 expression.[9] PXR can be activated by a variety of xenobiotic compounds, including rifampicin, nifedipine, and clotrimazole, as well as steroids, such as some glucocorticoids.[10,11] In a recent report (M-F. Yueh, M. Kawahara, and J. Raucy, unpublished data, August, 2004), the engineering of DPX-2 cells was described. This cell line is a derivative of the HepG2 cells but harbors human PXR and a luciferase-linked CYP3A4 promoter. These cells were used as a tool to evaluate the potential for rapidly and efficiently identifying new chemical entities for their ability to induce CYP3A4 at the cellular level.

The present investigation focused on characterization of several known inducers of the CYP3A family, such as rifampicin and omeprazole,[12] and their effects on CYP3A induction at both the transcriptional and enzyme activity levels in DPX-2 cells. CYP3A4 transcriptional activation in

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these cells was assessed by real-time polymerase chain reaction (PCR) and luciferase reporter gene activity. Further, we also assessed the effects of inducers on DPX-2 cells by performing a subsequent cytotoxicity assay. In addition, the ability of certain compounds to inhibit CYP3A4-mediated metabolism in rifampicin-induced DPX-2 cells was examined in reactions using Vivid fluorogenic substrates. These substrates have been previously applied in P450 metabolism and inhibition studies with heterologously expressed P450 enzymes.[13] Our results demonstrate that multiple assays can be performed in the DPX-2 cell line, allowing identification of CYP3A4 substrates and inducers and enabling in-depth characterization of their interactions with the CYP3A4 isozyme, including cytotoxicity. Similar approaches can also be used to create easy read-out assays for the assessment of multiple effects of P450 induction and inhibition for other inducible members of the human cytochrome P450 family.

Materials and Methods

Reagents

The Vivid CYP3A4 Blue Substrate was provided by Invitrogen Drug Discovery (Madison, WI). Resazurin was provided by Molecular Probes (Eugene, OR). The CYP3A4, CYP1A2, PXR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Light Upon eXtension (LUX) Fluorogenic Primers were provided by Dr Sandrine Javorschi at Invitrogen (Carlsbad, CA). Clotrimazole, dexamethasone, 5,5-diphenylhydantoin (phenytoin), ketoconazole, mevastatin, mifepristone, nifedipine, omeprazole, paclitaxel, rifampicin, troglitazone, troleandomycin, and verapamil were purchased from Sigma-Aldrich Corp (St Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, and Dulbecco's phosphate-buffered saline (PBS) containing Mg/Ca were obtained from Gibco (Grand Island, NY). Bovine growth serum (BGS) was purchased from Hyclone (Logan, UT).

Generation of the DPX-2 Cell Line

Stably integrated DPX-2 cells were constructed as described (M-F. Yueh, M. Kawahara, and J. Raucy, unpublished data, August, 2004). Briefly, cells were transiently cotransfected with an expression vector containing human PXR and with a modified luciferase vector harboring the CYP3A4 promoter and the distal and proximal enhancers. Cells containing both vectors were subjected to antibiotic selection and the surviving colonies were screened and purified.

Cell Culture Conditions

DPX-2 cells were grown as monolayer cultures in DMEM containing 50 U/mL penicillin, 50 μg/mL streptomycin, selection antibiotic, and 10% BGS at 37°C (5% CO_2). HepG2 cells were cultured in the same manner with media lacking the selection antibiotic. Prior to treatment with inducers, cells were seeded in white 96-well plates (ViewPlate-96, Perkin-Elmer, Boston, MA) at 10 000 cells/well in 100 μL of assay medium (DMEM with 10% BGS) and incubated overnight (16 to 24 hours).

Treatment of Cells With Chemicals

Drug stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted directly into assay medium. The final DMSO concentration of 0.1% was maintained in all dilutions. DPX-2 cells were plated in 96-well dishes and treated with selected inducers by replacing the medium in each well with 100 μL of medium containing an appropriate concentration of inducer or DMSO control; each condition was repeated in quadruplicate. After a 24-hour treatment, cell medium containing inducer or DMSO was removed and replaced with inducer or DMSO containing fresh medium and incubated an additional 24 hours.

RNA Preparation

DPX-2 cells were treated with 10 μM rifampicin, 100 μM omeprazole, or 0.1% DMSO for 48 hours. Following treatment, the assay medium was removed and cells were washed once with prewarmed PBS. Cells were then lysed and RNA isolated using the Micro to Midi Total RNA Purification System from Invitrogen according to manufacturer's instructions.

Quantitative Real-time Reverse Transcriptase PCR Assay

Total RNA (2 μg) prepared from treated and untreated DPX-2 and HepG2 cells was subjected to reverse transcription. Synthesis of cDNA was performed with Super Script III RNase H-reverse transcriptase (Invitrogen) along with RnaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen). Gene expression was analyzed by the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using FAM-labeled LUX primers for CYP1A2, CYP3A4, PXR, and GAPDH using Platinum Quantitative PCR SuperMix-UDG (Invitrogen). Samples for real-time PCR were prepared in triplicate. Quantitative values were obtained above the threshold PCR cycle number (Ct) at which the increase in signal associated with an exponential growth for PCR products is detected. The relative mRNA levels in each sample were normalized according to the expression levels of GAPDH. The efficiencies of the target and GAPDH were found to be equal (data not shown). An induction ratio (treated/untreated) was determined from the relative expression levels of the target gene using $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$). The average of the real-time PCR measurements was used to calculate the mean induction ratio for each gene.

Luciferase Assay for CYP3A4 Induction

Luciferase activity in DPX-2 cells was assayed using the Luclite Luminescence Reporter Gene Assay System from Perkin-Elmer. Following treatment with inducers, the assay medium was removed and cells were washed once with room temperature PBS. White adhesive backing was applied to the plate and 100 μL of PBS was then added to each well followed by 100 μL of luciferase assay reagent. After a period of 10 minutes, luminescent readings were taken on a GENios Pro instrument from Tecan (Durham, NC) using a 5-second integration time. Fold-induction was calculated as the luminescent readings obtained with treatment of inducer compared with treatment with 0.1% DMSO (control).

Vivid Fluorescent Assay for CYP3A4 Induction

CYP3A4 activity in DPX-2 cells was assayed using the Vivid CYP3A4 Blue Substrate, which was prepared as a 20-mM stock solution in acetonitrile. Following treatment of cells with inducers, the assay medium was removed and cells were washed once with warm PBS. Prewarmed PBS (50 μL) was then added to each well. To initiate the Vivid fluorescent assay, 50 μL of 100-μM Vivid CYP3A4 Blue Substrate diluted in warm PBS was added to each well resulting in a final concentration of 50 μM substrate and 0.5% acetonitrile. Fluorescent readings were monitored kinetically at 37°C over a period of 30 minutes using a Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 409 nm, an emission wavelength of 460 nm, and a wavelength cutoff of 455 nm. Activity was measured as the rate of fluorescent metabolite production over the course of the reaction. Fold-induction was calculated as the activity observed after treatment with inducer compared with treatment with 0.1% DMSO (control).

Cell Viability Assay in DPX-2 Cells

The cell viability assay was performed using the substrate resazurin, which was prepared as a 10-mM stock solution in DMSO, and used according to manufacturer's instructions. Following treatment with various chemicals, cells were washed once with warm PBS and 100 μL of fresh warm PBS was then added to each well. To initiate the viability assay, 20 μL of 30-μM resazurin diluted in warm PBS was added to each well for a final concentration of 5 μM resazurin and 0.05% DMSO. Fluorescence was measured using a Gemini XS plate reader at an excitation wavelength of 530 nm, an emission wavelength of 590 nm, and a wavelength cutoff of 570 nm. Cell viability was measured as the rate of fluorescent metabolite production over the course of the reaction.

Assessment of CYP3A4 Inhibition in DPX-2 Cells

Stocks (10 mM) of clotrimazole, ketoconazole, mifepristone, and nifedipine were prepared in DMSO, while stocks of verapamil were prepared in water. These compounds were then diluted with PBS. Prior to the inhibition assay, DPX-2 cells were treated for 48 hours with 10-μM rifampicin. Following treatment, the assay medium was removed and cells were washed once with 100 μL of warm PBS followed by the addition of 50 μL of warm PBS containing inhibitor or appropriate solvent control. To initiate the CYP3A4 inhibition assay, 50 μL of 40-μM Vivid CYP3A4 Blue Substrate diluted in warm PBS was added to each well for a final concentration of 20 μM substrate and 0.1% acetonitrile. Fluorescent readings were monitored kinetically at 37°C over a period of 40 minutes as described above. Activity was calculated as the rate of fluorescent metabolite production over the course of the reaction and was determined in triplicate for each inhibitor. Percent inhibition by each test compound was calculated as the ratio of the activity observed in the presence of test compound compared with no inhibitor (solvent controls).

Statistical Analysis

Correlations between 2 groups were analyzed by Pearson's correlation coefficient and Spearman's Rho (ρ). The statistical parameters were calculated using the Prism software package.

RESULTS

Identification of CYP3A4 Inducers in the DPX-2 Cell Line

A cell line stably integrated with human PXR (hPXR) and a luciferase construct containing the CYP3A4 enhancer was created as recently described (M-F. Yueh, M. Kawahara, and J. Raucy, unpublished data, August, 2004). Cells exhibiting the greatest induction with 10 μM rifampicin were selected for study and termed DPX-2. Initially, we tested and ranked the ability of certain drugs to induce CYP3A4 transcriptional activity and enzyme levels in DPX-2 cells. Cells were grown in 96-well plates and incubated for 48 hours in the presence of 10-μM concentrations of PXR ligands including rifampicin, nifedipine, troleandomycin, clotrimazole, mifepristone, mevastatin, omeprazole, troglitazone, paclitaxel, dexamethasone, and phenytoin (Figure 1). After 48 hours, the effects of these compounds on CYP3A4 expression and enzyme activity were evaluated by the luciferase assay (Figure 1A) and by a fluorescent assay with Vivid CYP3A4 fluorescent substrate (Figure 1B), respectively. Compounds were ranked by their ability to induce luciferase activity mediated by CYP3A4 enhancers above that observed in cells treated with medium containing 0.1% DMSO (control). The majority of drugs previously identified as CYP3A4 inducers also enhanced CYP3A4-mediated luciferase reporter gene activity in DPX-2 cells (Figure 1A). Although the induction

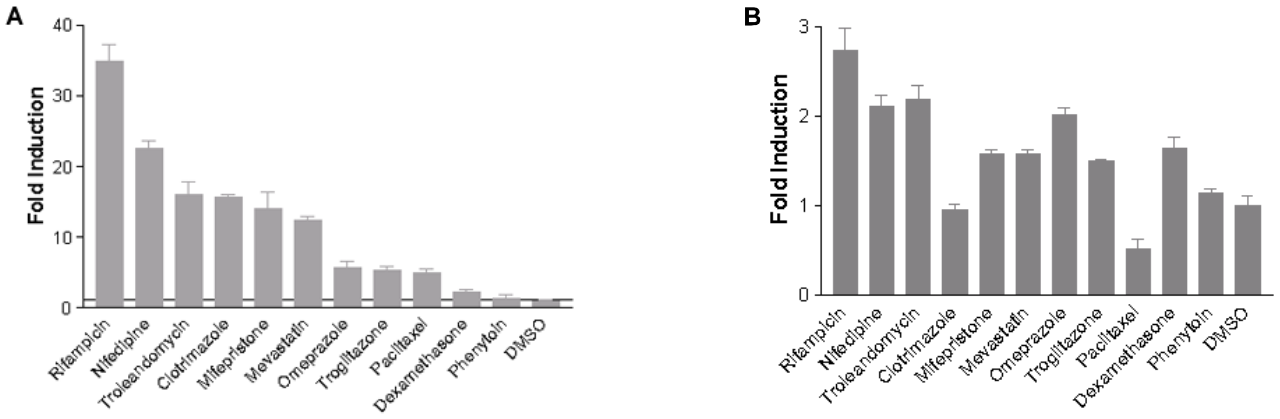

Figure 1. Effects of CYP3A4 inducers on luciferase activity in DPX2 cells. DPX2 Cells were treated with 0.1% DMSO or 10 μM of various compounds including rifampicin, nifedipine, troleandomycin, clotrimazole, mifepristone, mevastatin, omeprazole, troglitazone, paclitaxel, dexamethasone, and phenytoin for 48 hours. Following treatment, luciferase activity or CYP3A4 metabolism was assessed as described in Materials and Methods. Results are expressed as the fold-induction above DMSO (control) treated cells and is the mean of 4 determinations ± SE. Panel A are results generated in DPX2 cells using the reporter gene assay. Panel B represents CYP3A4 metabolism determined by using the Vivid assay.

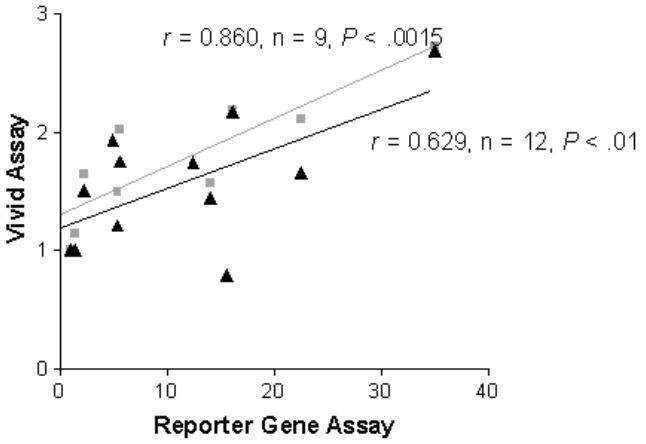

Figure 2. Correlation analysis between luciferase reporter gene assay and Vivid CYP3A4 enzyme activity. Correlations were measured by Pearson correlation coefficient (*r*) and Spearman's Rho (ρ). Analysis was performed (triangles) in the presence of all 12 test compounds, and (squares) in the absence of clotrimazole, paclitaxel, and omeprazole.

potency for compounds examined here varied significantly, most were consistent with previously reported data allowing discrimination between potent, moderate, and weak CYP3A4 inducers. For example, maximal levels of induction (up to 35-fold) were obtained for rifampicin, a previously reported prototypical CYP3A4 inducer of high potency,[14] whereas incubation in the presence of paclitaxel, a previously reported weak CYP3A inducer,[15] resulted in only a 2-fold induction of luciferase reporter gene activity.

In addition to assessing transcriptional activation by measuring reporter gene activity, we determined if these same compounds altered CYP3A4 enzyme activity in DPX2 cells by using Vivid CYP3A4 Blue fluorescent substrate (Figure 1B). In general, most drugs exhibiting induction in the luciferase-based reporter assay also increased enzyme activity in the Vivid assay. However, the increase for certain compounds was different when enzyme activity (Vivid fluorescent assay) was assessed. For example, drugs such as clotrimazole and paclitaxel did not exhibit any CYP3A4 induction in the enzyme assay, but enhanced CYP3A4-mediated gene activation. Conversely, based on a ranking order, omeprazole appeared to be a more potent inducer in the Vivid assay than in the luciferase reporter gene assay. In general, the correlation between the reporter gene assay and CYP3A4 activity was relatively poor, with a correlation coefficient of $r = 0.629$ (or $\rho = 0.479$, $n = 12$, $P < .01$) (Figure 2). The drugs clotrimazole, paclitaxel, and omeprazole represented discrepancies between the 2 assays. In the absence of these outliers, the correlation between the 2 assays was significantly improved with $r = 0.860$ (or $\rho = 0.900$, $n = 9$, $P < .0015$).

Effects of the Selected CYP3A4 Inducers on DPX-2 Cell Viability

In order to further explore the reasons for the differences in induction for certain agents between the enzyme activity and the reporter gene assays, we performed additional studies to determine the potential for cytotoxicity produced by the CYP3A4 inducers. For the majority of agents tested, treatment of DPX-2 cells with 10 μM concentrations of each drug did not produce a significant toxic effect (Figure 3A). Two exceptions were the drugs paclitaxel, a chemotherapeutic agent that is toxic to cells,[16] and mevastatin, a drug also associated with previously reported cytotoxic effects.[17] Upon treatment of DPX-2 cells with 10 μM of either paclitaxel or mevastatin, the number of viable cells was reduced by 70% and 15%, respectively. Consequently, the fold-induction determined for CYP3A4 activity in DPX-2 cells employing CYP3A4 Vivid Blue fluorogenic substrate was corrected for percent of viable cells (Figure 3B). After the correction for cell viability, mevastatin and paclitaxel exhibited enhanced CYP3A4 activity toward Vivid Blue analogous to that observed with the reporter gene assay. However, the induc-

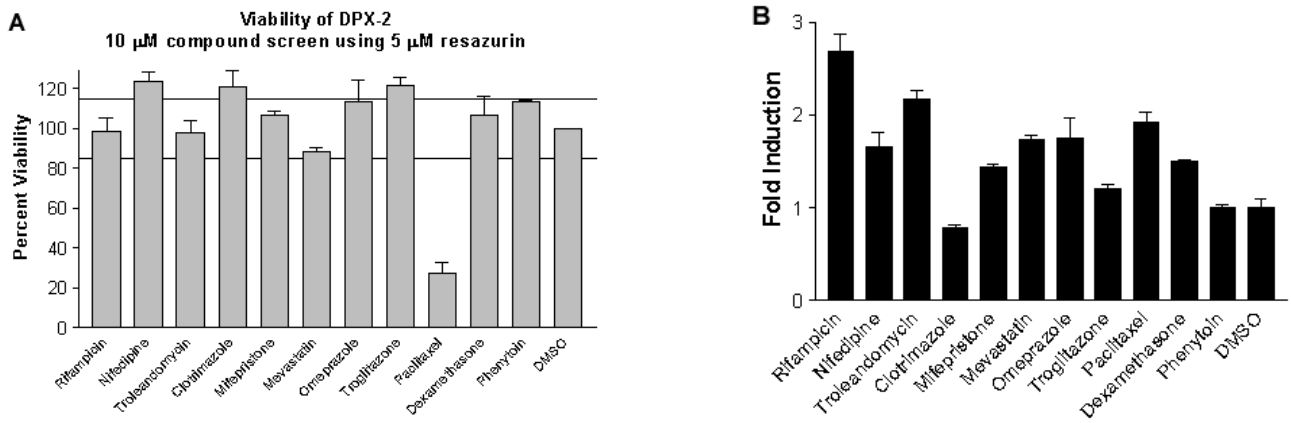

Figure 3. Viability screening of CYP3A4 inducers in DPX2 cells. Viability was assessed as described in Materials and Methods in cells treated for 48 hours with DMSO or 10 μM rifampicin, nifedipine, troleandomycin, clotrimazole, mifepristone, mevastatin, omeprazole, troglitazone, paclitaxel, dexamethasone, and phenytoin. In panel A results are expressed as viable cells (% of DMSO-treated cells) determined in quadruplicate ± SE. Panel B is the ranking of CYP3A4 inducers determined by the Vivid assay corrected for cell viability. Results are expressed as the fold-induction above DMSO-treated cells and is the mean of 4 determinations ± SE.

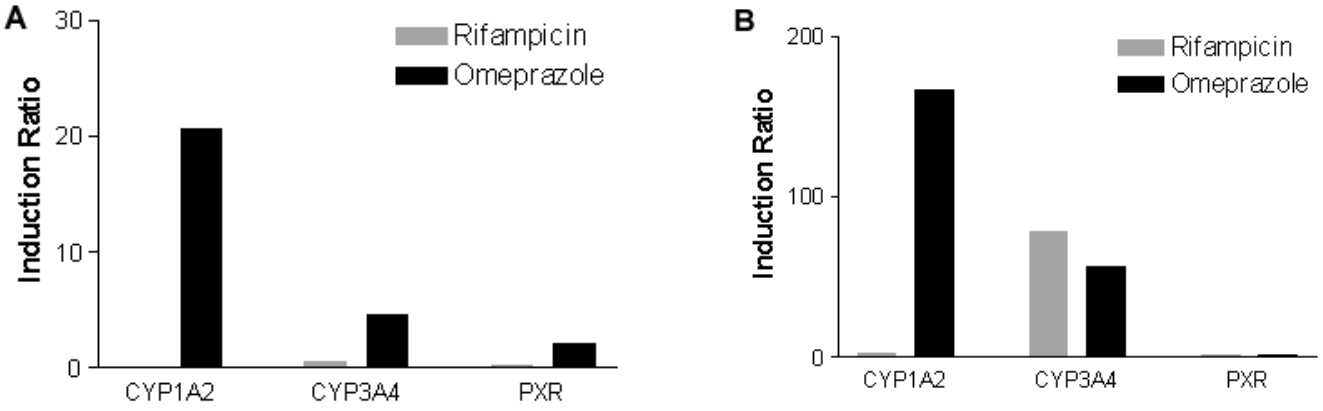

Figure 4. Quantitative changes in CYP3A4, CYP1A2, and PXR mRNA levels following induction with rifampicin and omeprazole. Cells were treated for 48 hours with DMSO, 10 μM rifampicin, or omeprazole. Following treatment, RNA (2 μg) was isolated and subjected to reverse transcriptase. Gene expression was analyzed by the ABI Prism 7700 sequence detection system as described in Materials and Methods. The relative mRNA levels were normalized to GADPH and the induction ratio was determined from the relative expression levels. Results are expressed as the induction ratio. Panel A, HepG2 cells. Panel B, DPX-2 cells.

tion potency of omeprazole and clotrimazole was not affected by the correction for cell viability in the Vivid assay and therefore required additional investigation.

Quantitative Changes in CYP3A4, CYP1A2, and PXR mRNA Levels Following Omeprazole Treatment

In addition to CYP3A4, omeprazole is known to induce other members of the cytochrome P450 family, including CYP1A isozymes.[18] Here we explored if omeprazole treatment of DPX-2 cells could also result in induction of other endogenously expressed P450s, such as CYP1A2. DPX-2 cells were treated with omeprazole followed by harvesting and isolation of RNA. CYP3A4, CYP1A2, and PXR mRNA levels were subsequently determined by quantitative real-time reverse transcriptase (RT)-PCR. Separate RT-PCR reactions were performed on RNA from the parental HepG2 cell line. In addition to omeprazole, rifampicin, the prototypic CYP3A4 inducer, was used as a positive control for CYP3A4 induction in DPX-2 and HepG2 cells (Figure 4). Rifampicin failed to increase CYP3A4, 1A2, or PXR levels in the parental HepG2 cell line (Figure 4A), but was a very potent CYP3A4 inducer in genetically engineered DPX-2 cells (Figure 4B). In addition, omeprazole treatment resulted in increased mRNA levels of CYP1A2 and CYP3A4 in both HepG2 and DPX-2 cell lines (Figure 4). The lack of hPXR enhancement in DPX-2 cells by omeprazole is most likely due to the high expression levels of the receptor produced by incorporation of hPXR into the genome. Omeprazole effects in DPX-2 cells were much more prominent, resulting in over 160-fold and 55-fold higher ratios for CYP1A2 and CYP3A4 respectively. Because Vivid CYP3A4 Blue fluorescent substrate has limited CYP3A4 selectivity with possible overlapping substrate specificity towards CYP1A2,[19] the overall increase in enzyme activity observed in the Vivid assay after omeprazole treatment could reflect a cumulative effect of endogenous CYP3A4 and CYP1A2 induction. The later observation

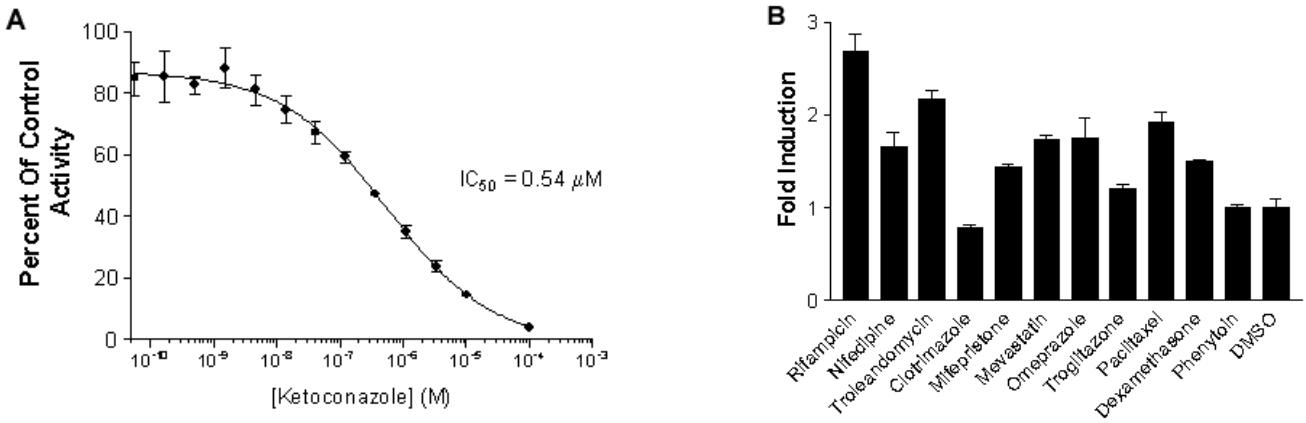

Figure 5. Comparison between IC_{50} values obtained in DPX2 cells and by recombinant CYP3A4. Inhibition of CYP3A4-mediated metabolism of Vivid by ketoconazole was determined in DPX2 cells and in BACULOSOMES containing heterologously expressed CYP3A4. DPX-2 cells cultured in 96-well plates were exposed to 10 μM rifampicin for 48 hours. Following induction, various concentrations of ketoconazole were incubated in the presence of 20 μM Vivid for 40 minutes. Values are expressed as the % of control rates (absence of inhibitor) and are the mean ± SE of 3 determinations. Panel A shows the results of varying the concentration of ketoconazole from 0.001 μM to 10 μM in DPX2 cells and Panel B shows results generated in BACULOSOMES using similar concentrations of ketoconazole.

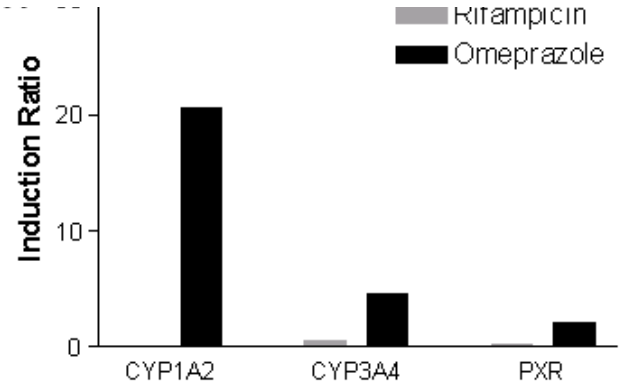

Figure 6. The effect of various inhibitors on CYP3A4-mediated metabolism of Vivid. Inhibition of CYP3A4 metabolism was monitored in the presence of various inhibitors including 10 μM ketoconazole, clotrimazole, mifepristone, nifedipine, and verapamil in DPX2 cells as described in Materials and Methods. Results are expressed as the percent of control activity (no inhibitor) and is the mean of 3 determinations ± SE.

may explain the higher induction observed for CYP3A4 catalytic activity when compared with the luciferase-based reporter gene assay.

Screening for CYP3A4 Inhibition in DPX2 Cells

One possible explanation for the inability of clotrimazole to induce CYP3A4 activity in the Vivid assay may be that this drug also acts as a potent inhibitor of CYP3A4 activity in DPX-2 cells. To further explore this possibility, we performed inhibition analysis of CYP3A4 activity in DPX-2 cells. DPX-2 cells were induced with 10 μM rifampicin for 48 hours followed by analysis of cytochrome P450 activity with Vivid CYP3A4 fluorogenic substrate in the presence of several drugs, including known CYP3A4 substrates and inhibitors. Ketoconazole, a prototypic CYP3A4 inhibitor,[20] was used as a positive control for CYP3A4 inhibition (Figure 5). Initially, we compared the inhibitory potency of ketoconazole in DPX-2 cells (Figure 5A) with another well-established in vitro assay employing recombinant CYP3A4 expressed in BACULOSOMES (Figure 5B). In both assays, ketoconazole appeared to be a potent inhibitor of CYP3A4 activity, with apparent concentrations required to produce 50% inhibition (IC_{50}) values in the submicromolar range. Nevertheless, apparent IC_{50} values obtained for ketoconazole in a cell-based assay were higher (0.54 μM) compared with IC_{50} values obtained in BACULOSOMES (0.04 μM). One possible explanation for the lower inhibitory effect of ketoconazole in the DPX-2 cell-based assay is that cell membrane permeability and transport may result in lower intracellular concentrations of the inhibitor compared with BACULOSOMES that are in a homogeneous cell free format. Despite the difference in actual IC_{50} values, 10 μM ketoconazole acted as a potent inhibitor of CYP3A4 activity in DPX-2 cells, inhibiting up to 85% of rifampicin-induced cytochrome P450 activity (Figure 6). In addition to ketoconazole, clotrimazole demonstrated a similar inhibitory effect in rifampicin-induced DPX-2 cells producing up to 75% inhibition of CYP3A4 control activity (Figure 6). When CYP3A4 metabolic rates were determined in the presence of 3 other agents, nifedipine, mifepristone, and verapamil, inhibition, activation, or a combination of both activation and inhibition of Vivid metabolism in DPX-2 cells was observed. Both mifepristone and nifedipine are known CYP3A4 substrates; therefore, in the fluorescent Vivid reaction these compounds exhibited competitive inhibition, reducing the reaction rates by ~40%. Interestingly, the presence of 10 μM verapamil, another known CYP3A4 substrate, resulted in a 3-fold activation of the Vivid reaction rate (Figure 6). This complex CYP3A4 kinetic mechanism was reported previously and included evidence that CYP3A4 can bind and metabolize multiple substrate molecules simultaneously.[21] Verapamil is one CYP3A4

substrate with atypical kinetic parameters. Indeed, biphasic substrate inhibition and activation for different effectors, including verapamil, were observed in assays employing enriched microsomal fractions expressing recombinant CYP3A4.[22] Therefore, the marked elevation in Vivid activity observed in rifampicin-induced DPX-2 cells in the presence of verapamil could be viewed as a further extension of the previous observation, but demonstrating activation of CYP3A4 metabolic rates at the cellular level.

DISCUSSION

Currently, significant effort by many early absorption, distribution, metabolism and excretion (ADME) programs is being devoted to identifying compounds that may alter the major cytochrome P450 oxidative pathways involved in drug metabolism.[23,24] Alterations in these pathways may stem from inhibition or induction of the human P450 enzymes. Identification and evaluation of the relative potencies of these inhibitors and inducers that alter metabolic rates can enhance the safety of new chemical entities. Of the P450 enzymes, CYP2D6 and CYP3A4 are of particular importance due to their pivotal roles in drug metabolism.[25] CYP3A4 is involved in a significant number of drug-drug interactions leading to adverse drug reactions and toxicity stemming from alterations in activity and expression.[26,27] Screening for ADME properties of new chemical entities, and in particular, identifying compounds that are potent CYP3A4 inhibitors, is now a widely accepted part of the modern drug discovery process that is commonly employed by the pharmaceutical industry at different stages of drug discovery and development.[28,29] At the same time, the ability to identify compounds that are potent inducers of CYP3A4 activity remains less investigated, partially due to the lack of mature technology.

Until recently, primary cultures of human hepatocytes were the major tools to investigate CYP3A4 induction.[30] However, one of the obstacles to using primary human hepatocytes is the interindividual donor variability in P450 expression and catalytic rates. Furthermore, isolated hepatocytes exhibit a rapid loss of P450 expression after a short time in culture.[31,32] Presently, cell-based PXR reporter gene assays to screen CYP3A4 inducers have been reported as an alternative approach to screen for P450 induction.[12,33,34] Results generated in these reporter gene assays were compared with those obtained in primary cultures of human hepatocytes[33] and revealed a significant correlation between the 2 systems. However, additional efforts were not employed to investigate the ability of certain drugs to exhibit multiple, synergistic, or antagonistic affects on CYP3A4 metabolism at the cellular level. Moreover, selected drugs can act simultaneously as P450 substrates, inducers, and inhibitors and, in some cases, can regulate their own metabolism.[35] The latter findings further complicate the assessment of drug-drug interactions necessitating the application of several independent methods to evaluate multiple drug effects. Recognizing whether drugs act as CYP3A4 substrates, inducers, or inhibitors is instrumental in discriminating between different mechanisms involved in drug-drug interactions. Furthermore, identifying the mechanism allows an evaluation of overall metabolic and toxic responses and in better predicting pharmacokinetic parameters for in vivo drug administration.

Here, we demonstrated that DPX-2 cells, harboring human PXR and a luciferase-linked CYP3A4 promoter element, present a suitable system to investigate these multiple effects. Results obtained by the reporter gene assay on CYP3A4 induction in DPX-2 cells and those obtained by assessing enzyme activity were generally similar as demonstrated by correlation analysis. However, possible outliers were also identified. The correlation coefficient was markedly improved from $r = 0.629$ to $r = 0.860$ ($P < .0015$) when 3 outliers were excluded from the set. Subsequently, we investigated possible reasons for these outliers by considering other aspects of cell physiology and metabolism. Thus, we included cell viability assessment and enzyme inhibition assays at the cellular level. Our results demonstrated that several drugs, initially identified as CYP3A4 inducers in the luciferase reporter assays, may also exhibit additional effects, acting as toxic agents (mevastatin and paclitaxel) affecting cell viability, or as inhibitors of rifampicin-induced CYP3A4 activity (clotrimazole). In addition, drugs such as omeprazole can act as potent inducers for more than one cytochrome P450 isozyme. In results presented here, omeprazole exhibited multi-isozyme inductive properties by enhancing expression of both CYP3A4 and CYP1A2 as determined by RT-PCR reactions. Induction of both P450s by omeprazole revealed a cumulative effect on metabolism observed by the markedly increased catalytic activity in DPX-2 cells.

CONCLUSION

Results presented here demonstrated that several independent assays could be used in a complementary fashion in DPX-2 cells to gain a better understanding of various underlying aspects of altered rates of drug metabolism by CYP3A4. Collectively, our approach presents an attempt to systemically investigate complex drug effects on multiple aspects of CYP3A4 metabolism involving both inhibition and induction. In the future, similar systematic approaches employing other P450 enzymes could be applied to investigate complex mechanisms allowing more complete and careful assessment of in vivo metabolic reactions.

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